UNIVERSITI PUTRA MALAYSIA

Cytotoxic Effect of Betulinic Acid on Vascular Smooth Muscle Cells.

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By

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Thesis submitted to the School of Graduate Studies,University Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science.

Cytotoxic Effect of Betulinic Acid on Vascular Smooth Muscle Cells.

By

RAJA KUMAR VADIVELU
April 2009

Chairman : Assiciate Professor Dr Abdul Rahman Omar, PhD
Faculty : Institute Bioscience

Betulinic Acid (BA) is a widely available plant-derived triterpene reported as a selective cytotoxic activity against cancer cell of neuroectodermal origin and leukaemias. Interestingly, this will be first report to demonstrate the cytotoxic effect of BA in VSMCs. First, MTT cytotoxic assay was used to measure cell viability of VSMCs with predetermined concentrations of BA for 24h, 48h and 72h. The results obtain indicated that BA inhibit the growth and proliferation of VSMCs in a dose dependent manner IC_{10} of 0.4µg/ml, IC_{25} of 1µg/ml and IC_{50} of 3.8 µg/ml significantly ($P<0.05$). Secondly, the genotoxic potential associated to exposure to BA was assessed on VSMCs *in vitro* by the comet assay. BA exhibits low level of DNA damage and not likely to increase the level of DNA damage after 24 h exposure. Moreover, Flow cytometric analysis revealed that BA treatment FOR 24 h induced cell cycle arrest at the G1 phase and caused the appearance of a sub-G1 DNA peak at 48 h. Finally, the cell death morphology indicates that the percentage of apoptotic at 24 h were 12.43 ± 1.55% and at 48 h were 23.17 ± 1.73%, the percentage of necrotic cells at 48 h were 14.63 ± 1.45%. An increase percentage of apoptotic cells at 72 h were 45.92 ± 1.45%. In conclusion, exposure of BA to VSMCs initiate early DNA damage, arrest at G1 phase and induce apoptosis.

Abstrak tesis yang dikemukakan kepada Senat University Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

Kesan Sitotoksik Betulinic Acid terhadap Sel Otot Licin Vaskular.

Oleh

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Betulinic Acid (BA) merupakan extrak triterpene daripada tumbuhan yang menunjukan aktiviti sitotoksik terhadap sel kanser neuroekotoderma dan lekumia. Ini ialah kajian pertama yang meneliti kesan sitotoksik terhadap cell otot licin vascular (VSMCs). Peneletian assai sitotoksik MTT pada masa 24, 48 dan 72 jam menunjukkan nilai dos BA yang merencat pertumbuhan dan proliferasi VSMCs ialah IC_{10} of 0.4µg/ml, IC_{25} = 1µg/ml dan IC_{50} = 3.8 µg/ml pada aras signifikan ($P<0.05$). Sementara itu BA menunjukkan kesan genotoksik yang kurang pada masa 4 jam dan tidak meningkatkan kesan genotoksiknya pada masa rawatan 24 jam. BA juga merencat kitaran sel pada fasa G_1dalam tempoh 24 jam dan meningkatkan fasa sub-G pada 48 jam, kajian ini dikenalpasti dengan menggunakan teknik sitometri aliran. Akhirnya, peratus kematian sel pada 24 jam didapati 12.43 ± 1.55% dan pada 48 jam ialah 23.17 ± 1.73%, manakala peratus kematian cell melalui proses nekrotic ialah 14.63 ± 1.45% pada 48 jam. Seterusnya, peningkatan peratus kematial cell melalui proses apoptosis didapati 45.92 ± 1.45% pada masa 72 jam. Kesimpulanya, kesan BA terhadap VSMCs menunjukan kerosakan DNA pada peringkat awal dan seterusnya mencetus serangan cell pada fasa G1 dan diikuti oleh kematian sel melalui proses apoptosis.

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I certified that an Examination Committee met on 1st April 2009 to conduct the final examination of Raja Kumar Vadivelu on his Master of Science thesis entitled "Cytotoxic effect of Vascular Smooth Muscle Cells (VSMCs)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981.The committee recommends that the candidate be awarded the relevant degree. Member of committee are as follows:

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This thesis submitted to the Senate of the Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 16 October 2009

DECLARATION

I hereby declare that the thesis based on my original work except for quotation and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for another degree at UPM or other institutions.

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RAJA KUMAR VADIVELU

Date: 16 September 2009

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4EBP-1	*-Eukaryotic translation initiation factor 4E binding protein.*
AO	*-Acridine orange*
BA	*-Betulinic Acid*
bFGF	*-Basic fibroblast growth factor*
BrdU	*-Bromodeoxyuridine*
BSA	*-Bovine serum albumine*
CABG	*-Coronary Arthery bypass Graft*
CDKs	*-Cyclin Dependent Kinase*
CDKi	*-Cyclin Dependent kinase inhibitor*
Cox-2	*-Cyclooxygenese -2*
DNA	*-Deoxyribonucleic acid*
DMSO	*-Dimethyl Sulfoxide*
ECM	*-Extracellular matrix*
EDTA	*-Ethylene diamine tetraacetic acid*
EGF	*-Epidermal growth factor*
EGF-R	*-Epidermal growth factor - receptor*
elf2B	*-Eukaryotic initiation factor 2B*
ERK1/2	*-Extracellular- signal regulated kinase 1/2*
JNK	*-c-Jun N-terminal kinase*

MAPK	*-Mitogen activated protein kinase*
MCP-1	*-Monocyte chemoattractant protein 1*
m	*-Milli*
M	*-Molar*
Mdm2	*-Mouse double minute 2*
MMP	*-Matrix Metalloprotenase*
mTor	*-Mammalian target of rapamycin*
MTT	*-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
MPT	*-Mitochondria permeability transition*
NFκB	*-Neclear factor κ B*
PARP	*-Poly (ADP – Ribose) prolimerase.*
PBS	*-Phospate Buffered Saline*
PCNA	*-Proliferating Cell Nuclear Antigen*
PI	*-Propodium Iodide*
PI3-k	*-Phosphoinositide-3 kinase*
POBA	*-Ballon Coronary Angioplasty*
PTCA	*-Precutanueous Transluminal Coronory Angioplasty*
Rb	*-Retinoblastoma protein*
RNA	*-Ribonecleic acid*
S6K1	*-Ribosomal S6 kinase 1*
SmBM	*-Smooth Muscle Basal Media*
ISR	*-In Stent Restenosis*
VSMCs	*-Vascular smooth Muscle Cells*
µ	*-Micro*

CHAPTER 1

1 INTRODUCTION

1.1 Background

Cardiovascular diseases continue to be the major hospital admissions and death in government hospitals in Malaysia (Anas *et al*., 2008). The trend in the prevalence of coronary artery disease (CAD) associated with artheriochelorosis is believed to be increasing in Malaysia although limited epidemiological data are presently available. According to Khoo et al, (1991) cardiovascular diseases occupied third place as the cause of death from 1950 to 1989. An ethnical based study from Chiam et al, (2002) indicates that the prevalence of combinations of hypertension and hyperlipidaemia among patients undergone coronary artery bypass grafting (CABG) in Hospital Universiti Kebangsaan Malaysia were 46.0% Chinese, 40.1% Malays and 11.6% Indians. Recently, Shahar et al, (2007) indicated that almost 80% of Malaysian men have higher total cholesterol and LDL-cholesterol and at high risk of CAD.

Atherosclerosis involves the proliferation of smooth muscle cells and the accumulation of lipids and other major causative factors in the pathogenesis of coronary artery disease (CAD) and restenosis after angioplasty. The widely used treatment for CAD complication is Percutaneous Transluminal Coronary Angioplasty (PTCA), which was employed to enlarge obstructed coronary artery. However, overshadowing of PTCA was the major cause of angiographic restenosis and periodically similar symptoms at 6 months. Ozaki et al,1996 estimated restenosis rates at 12.7%, 43%, 49.4% and 52.5% at 1, 3, 6 and 12 months respectively. This occurrence is due to the adverse effect of PTCA procedure

which cause lesion to the aerial wall. Injured arterial wall secretes procrine and autocrine molecules which alter the cell cycle phase and phenotypic structure of Vascular Smooth Muscle Cells (VSMCs). The proliferation and migration of VSMCs are the major attributes for the narrowing of the artery lumen. This occurrence is described as restenosis.

Intracoronary stent was introduced to reduce the rate of angiographic and clinical restenosis compared to percutaneous transluminal coronary angioplasty (PTCA) alone. These methods imply by using a "stent" a metal bar accompanied together with PTCA procedure in odder to impede thinking of the neointima layer of the artery. However, stent placement also contributes to in-stent restenosis. New tissues favorably grow inside the stent because the stent is placed rigidly attached to blood vessel. Initially, this new tissue consists of healthy VSMCs and endothelium cells from the lining of the arterial wall. This is a constructive effect because development of normal lining over the stent allows blood to flow smoothly over the stented area without clotting. However, in about 25% of patients the appearance of scar underneath the lining of the artery may become so thick that it can obstruct the blood flow and produce an important blockage. In-stent restenosis is typically seen 3 to 6 months after the procedure; after 12 months have passed uneventfully, it is rare.

Recently, an improved outcome with using an attractive alternative to bare-metal stents is drug eluting stent. This is a promising new treatment involve coating the outer aspect of a standard coronary stent with a thin polymer containing medication that can prevent the formation of scar tissue at the site of coronary intervention. Currently, two drug-eluting stents used widely are Sirolimus-Eluting Balloon, and Paclitaxel Eluting Stent (TAXUS)-IV. Both These techniques demonstrated striking reductions in angiographic restenosis and revascularization rates with sirolimus- and paclitaxel-eluting stents, respectively. However, comparative clinical trials have shown that drug eluting stent do not confer any benefit in

clinical outcomes (Grube *et al*., 2002) and may even predispose to stent thrombosis (Rhee *et al*.,2008). Furthermore, the impediment to the widespread adoption of drug-eluting stents is the high cost (Bakhai *et al*., 2003). Therefore, an alternative method the prevent restenosis after PTCA will be interesting.

In the last decade the implication of natural compounds in controlling the proliferation and migration of neointima in diseased arthery has become evident. Alternative to Paclitaxel and Sirolimus, the search of other natural substances will become a useful for the treatment of restenosis by prevailing over the complications and obstruction of DEC application. Interestingly, there are various natural compounds such as Emodin (Heo et al., 2008), Raphanus sativus extract (Suh *et al*., 2006), that have great potential to inhibit the growth of VSMCs and induce apoptosis. Although DES limits restenosis, adverse vascular pathologies and toxicities continue to be of major concern. Higher concentration of paclitaxel may lead to increased apoptosis in the vessel wall and consequently to a more unstable phenotype of the pre-existing atherosclerotic lesion (Shishehbor *et al*., 2008). Whereas, sirolimus-eluting stents was not shown to effect on arterial pathology but it was described temporarily lead to systemic concentrations that approach immunosuppressive levels (Sousa *et al*., 2001). Thus, application of a non toxic anti proliferative and anti migratory compound will be interesting to prevent restenosis.

In this regard, the Betulinic acid (BA) (3β-hydroxy-lup-20(29)-en-28-oic acid), a pentacyclic lupane triterpene, has shown potential in inducing cell cycle arrest and apoptosis in various cancer cell. Convincingly, BA has advantages such as non toxic compound (Alakurtti *et al*., 2006) with ability to decrease *cyclin D1* and *Bcl* gene expression, and induce apoptosis through p53-independent pathway. Thus, BA is considerately involved to inhibit VSMCs proliferation, migration and induce apoptosis.

Vascular smooth muscle cells (VSMCs) are the prime cellular component of the normal artery as well as of intimal lesions that develop in response to arterial injury. Consequently, proliferation and migration of VSMCs are hallmarks of vascular disorders such as atherosclerosis and restenosis (Dzau *et al.*, 2002). The purpose of this study was to investigate the effects of BA on VSMC proliferation and migration. To achieve these aims VSMCs cultures were used to access the cytotoxicity, potential of growth inhibition, genotoxicity and induction of apoptosis by BA.

1.2 Objective of the study

The aims of the present study were, first, to elucidate whether BA is able to attenuate VSMC growth and proliferation *in vitro*.

Specifically, the present thesis aimed at

- To study the potential of BA to reduce cell viability and inhibit growth arrest.
- To evaluate the level of genotoxic effect of BA in VSMCs.
- To elecidate the mode of cell cycle arrest.
- To confirm the mode of cell death.

CHAPTER 2

2 LITERATURE REVIEW

2.1 The Percutaneous Transluminal Coronary Angioplasty

Percutaneous Transluminal Coronary Angioplasty (PTCA) has been established since 1978 by Andreas Gruentzig (Landzberg *et al*., 1997) for treating progressive atherosclerosis which leads to the formation of fibrous plaques and accompanied by narrowing of artery. This technique is widely used as a non-surgical revascularization procedure that involves opening obstructed coronary arteries for patients with coronary artery disease. It provides feasible alternative to coronary artery bypass graft (CABG) surgery as shown in Figure 2.1.

The physical force exerted by the inflated balloon catheter on the artery wall causes distention of the vessel resulting in luminal diameter gain and improved vessel patency and hence the blood flow to the myocardium (Libby. 2002). Despite of substantial clinical and economical advances in PTCA, the major limitation of this procedure is restenosis of the target vessel, manifesting as late arterial renarrowing at the site of intervention. Several studies indicated that restenosis rate were 15%, (Lokito *et al*., 2000) and 27%, (Cultip. 2006) during first 6 months after PTCA, other complications were reported as rapid closure and acute closure that can often lead to myocardial infarction, the need for emergency bypass surgery, or even death. Therefore, a metallic stent can be placed in the artery after balloon dilatation, in which case intensive anti platelet therapy and low dose aspirin is required for four weeks after the procedure (Gaspardone *et al*., 2005). This coronary stents procedure has not shown a promising impact on the clinical effectiveness, in reducing

abrupt vessel closure and restenosis. The stent is a foreign object and it stimulates an immune response. This may cause scar tissue (cell proliferation) to rapidly grow over the stent. In addition, there is a strong tendency for clots to form at the site where the stent damages the arterial wall.

The excessive scar tissue in the luminal wall of the coronary artery, results in neointimal hyperplasia (proliferation and migration of vascular smooth muscle cells and production of extracellular matrix). Coincidentally, the long term clinical outcome of stent implantation continues repeat renarrowing by a process called in stent restenosis ISR (Pendyala *et al*., 2008). Clinically ISR is still unacceptably high and has been shown to occur in 15% to 30% of people implanted with bare metal stents. The increase of extracellular matrix formation appears to form the bulk of the neointimal hyperplasia tissue in the lumen. Currently, prevention of ISR is accentuated by producing a stent that has potential to initiate the tissue response or any such pretreated stents need to be safe, user friendly, and not to add significantly to the cost or time required for the procedure. Interest has focused on ablative techniques as an alternative to balloon angioplasty (Figure 2.2).

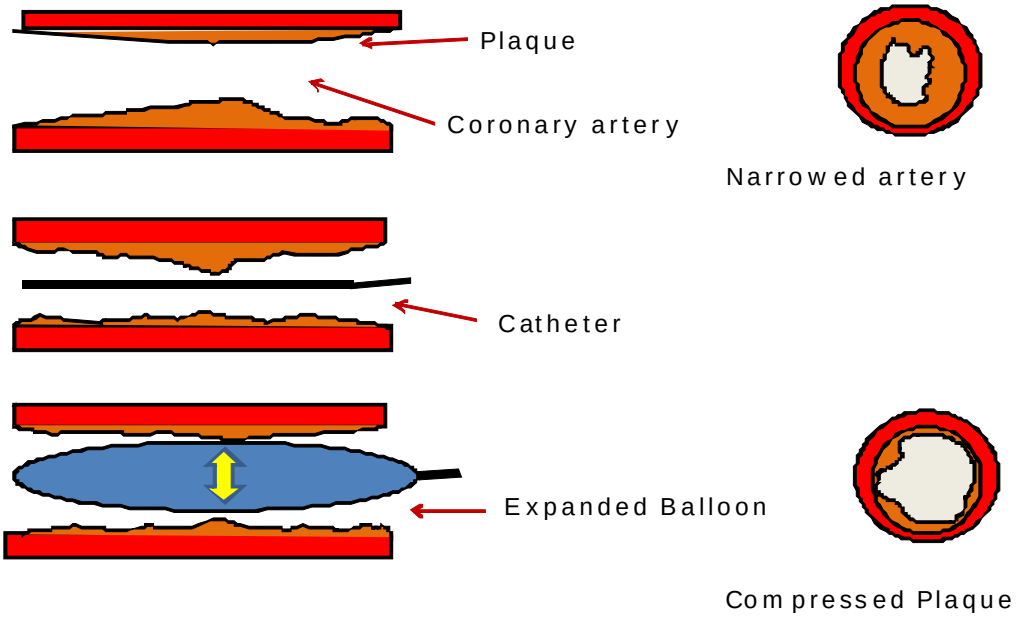

Figure 2.1 Plaque buildup artery is deflated with balloon catheter.

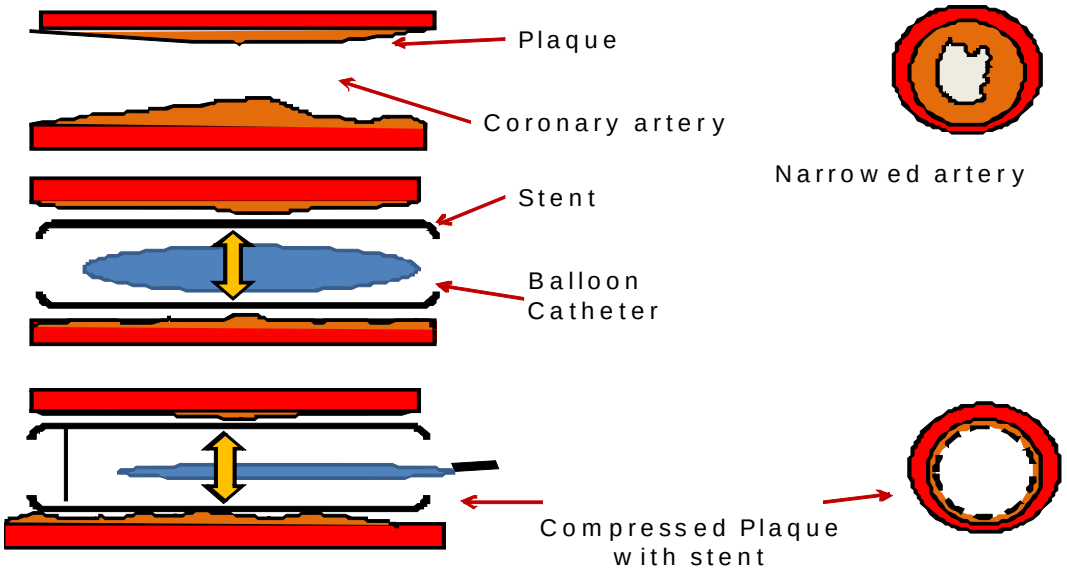

Figure 2.2 Restenosis of a stent-widened coronary artery.The illustration shows the restenosis of a stent-widened coronary artery. With expanded stent compresses plaque, allowing normal blood flow.

Recently, drug-eluting stents were designed to lessen this problem; by coating an antiproliferative drug can sucessfully avoid this in-stent restenosis. The first successful Drug-eluting stent approved by Food and Drug Administration (FDA) was sirolimus-eluting stents in 2003 (Thompson. 2003). Soon thereafter a series of trials of paclitaxel-eluting stents led to FDA approval of the Taxus stent in 2004 (DeJohn. 2004).

Interestingly, (DES) procedure associated with antithrombus therapies has dramatically decreased the mortality and morbidity in patients and have resulted in fairly low restenosis rates of <5% (Medina and Foto. 2004). The strategies to reduce in-stent restenosis by employing DES can be categorized into 3 processes: (1) inhibition of cell cycle entry by cytostatic drug with focus in arresting cell cycle at Go/G_1; (2) cytotoxic strategies, which induce the death of cells that enter the cell cycle but not necrosis ;(3) paracrine strategies, which inhibits down stream signiling involve in the progression of restenosis, such as antithrombosis and re-endothelialization, and inhibits vascular smooth muscle cell (VSMC) proliferation (eg, inhibiting TNF-α). At present, rapamycin and paclitaxel are the most clinically proven and predominantly used drugs in DES. They show promising evidence in inhibits cell cycle progression at the late G1 and M phases, respectively (Wessely *et al*., 2007).However, comparative clinical trials have shown no difference in patient outcomes, and drug-eluting stents are considerably more expensive than their bare-metal counterparts. Furthermore, observational clinical studies have shown that patients with diabetes have less favorable results after percutaneous coronary intervention compared with the non diabetic counterparts, but its mechanism remains unclear.

2.2 Mechanism of In-Stent Restenosis (ISR)

2.2.1 The arterial wall

Healthy arteries typically consist of three layers, the *tunica intima, tunica media* and *tunica adventia*. The intima comprises a continuous monolayer of tightly connected endothelial cells lying on a basement membrane within an interstitial matrix containing laminin and heparin sulphate proteoglycans. Tunica media is the thickest layer of the blood vessel and is comprised of vascular smooth muscle cells (VSMC), elastin, collagen, and other extracellular matrix components. The adventitia comprises loose connective tissue, capillaries, fibroblasts and fat cells separated from the outer media by an external elastic lamina.

2.2.2 Cellular events following angioplasty

Overstretching of the artery during balloon angioplasty and stenting results in dissection of the arterial media, endothelial damage, and fracturing of the arterial internal elastic lamina. Additionally, arterial lesion reflects a cascade of molecular and cellular events within the vascular wall which results in the release of numerous vasoactive, thrombogenic, cytokines and mitogenic factors. These phenomena involve both an acute and chronic phase. The early event of acute phase is elastic recoil generally occuring within 24 hours after mechanical streching. In the chronic phase (3-6 months) involves arterial modeling, Thrombus formation and neointima hyperplasia. However, with balloon coronary angioplasty (POBA) and procedure those do not involve stent in an acute event result reduction of lumen diameter very rapidly.